Scanning Microscopy

Volume 1 | Number 2 Article 25

12-30-1986

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Scanning Microscopy, Vol. 1, No. 2, 1987 (Pages 695-704)
Scanning Microscopy International, Chicago (AMF O'Hare), IL 60666 USA

0891-7035/87$3.00+.00

LIGHT AND ELECTRON MICROSCOPIC LOCALIZATION OF GLYCOCONJUGATES WITH GOLD-LABELED REAGENTS

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(Received for publication May 30, 1986, and in revised form December 30, 1986)

Abstract

This paper gives an overview of the use of lectins, glycoproteins, monoclonal anti-carbohydrate antibodies, toxins, and glycosyltransferases for the detection and localization of cellular glycoconjugates in sections from embedded tissues by light and electron microscopy. In all these techniques particles of colloidal gold were used as a marker. For light microscopic studies routine conditions of embedding such as paraffin or Epon embedding could be employed. For electron microscopic studies the low temperature embedding technique using Lowicryl K4M was found to be the method of choice for the localization of sugar residues in intracellular compartments, the extracellular matrix and the plasma membrane. The applications of the various techniques will be illustrated with data about subcellular sites of certain glycosylation steps as well as the detection of glycocalyx domain formation and cellular heterogeneity in glycoclayx composition.

Key Words: Lectin-gold, glycoprotein-gold, cholera toxin-gold, monoclonal anti-carbohydrate antibodies, galactosyltransferase, glycocalyx, glycosylation, Golgi apparatus.

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Introduction

For the visualization of cellular and extracellular glycoconjugates at the light and electron microscopic level various staining reactions are available (Martinez-Palomo, 1970; Winzler, 1970; Rambourg, 1971; Geyer, 1973, 1977; Spicer et al., 1981). These techniques are based mainly on the detection of carboxyl and sulfate groups or periodic acid reactive configurations. Intrinsic problems with these techniques are the limitations in their specificity for particular saccharide residues and spatial resolution as well as that only a few of them can be used at the electron microscopic level for the detection of carbohydrates in intracellular compartments and in compact tissues (Wetzel et al., 1966; Rambourg et al., 1969). A more differentiated and specific approach was achieved by the use of lectins that bind to defined sugar sequences of oligosaccharide chains of glycoconjugates (Lis and Sharon, 1973; Nicolson, 1974; Goldstein and Hayes, 1978; Roth, 1978). Similarly, monoclonal antibodies against well characterized carbohydrate epitopes and certain toxins should provide highly specific tools. On the other hand, glycosyltransferases based on their strict acceptor specificities have already been shown to be valuable reagents in the biochemical elucidation of oligosaccharide structures (Sadler et al., 1979; Beyer et al., 1981). In the following the use of the specificity of the above mentioned reagents for the light and electron microscopic detection of glycoconjugates will be demonstrated. It should be emphasized that the electron microscopic studies mentioned here would not have been possible without the use of particles of colloidal gold as a marker and the application of the recently developed low temperature embedding technique with Lowicryl K4M (Carlemalm et al., 1982).

Studies with the use of lectins

Lectins are a family of multivalent carbohydrate binding proteins of plant and animal

origin which have been extensively used in the various fields of cell biology (Nicolson, 1974; Roth, 1978). Because of their ability to specifically recognize and bind sugar residues (Goldstein and Hayes, 1978; Lis and Sharon, 1973; Kornfeld and Kornfeld, 1978) they have found particular interest in carbohydrate cytochemistry and their validity as probes for in situ studies has been extensively and unequivocally demonstrated (Nicolson, 1978; Roth, 1978). For use in microscopical localization studies lectins must be linked to appropriate markers. In addition to a variety of other markers, particles of colloidal gold have proven to be particularly useful. Earlier studies performed with lectin-gold complexes have focussed on the detection of binding sites on the surface of cultured or isolated cells and aspects of their dynamic state (Horisberger et al., 1975; Horisberger and Vonlanthen, 1978; Wagner and Wagner, 1976; Wagner et al., 1976; Roth and Wagner, 1977a,b; Roth and Binder, 1978). More recent applications were aimed at the visualization of sugar residues in intact tissues and inside cells. This goal could be relatively easily achieved in light microscopy (Roth, 1983b; Lucocq and Roth, 1984, 1985) but is a much more difficult task in electron microscopy mainly due to the limited penetration of the reagents (Bretton and Bariety, 1974; Wood et al., 1974; Gros et al., 1977; Sato and Spicer, 1982). This problem can be circumvented by the staining of ultrathin frozen sections (Griffiths et al., 1982) or with the so-called freeze fracture cytochemistry (Pinto da Silva et al., 1981; see also the contribution of Pinto da Silva in this volume). Moreover, we could show (Roth, 1982,(1983b) that lectin-gold complexes can be efficiently used to label thin sections from low temperature Lowicryl K4M (Carlemalm et al., 1982) embedded tissues. This is illustrated in the following section.

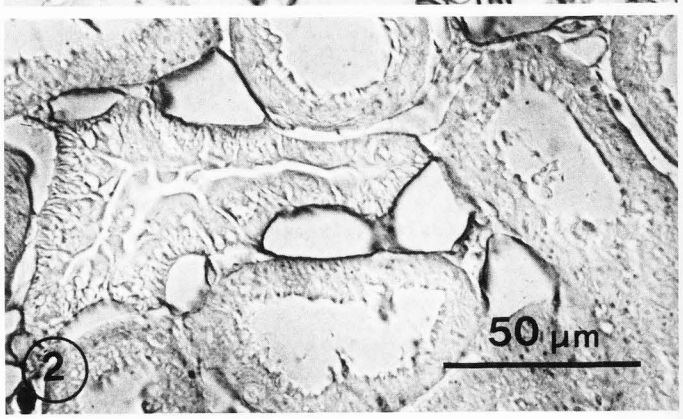
Methodical aspects

The preparation of particles of colloidal gold and the conditions for complex formation with lectins or glycoproteins are described in full detail in our earlier publications (Roth, 1983a,b,c; Roth et al., 1984a; Lucocq and Roth, 1985). The complex formation of proteins with colloidal gold is pH dependent (Geoghegan and Ackerman, 1977). In Table 1 the pH values for complex formation of lectins and glycoproteins with colloidal gold are listed. We have estimated with the salt flocculation test (Zsigmondy, 1901; Roth and Binder, 1978) the optimal amount of lectins or glycoproteins needed for the stabilization of a certain volume of colloidal gold (Roth, 1983b,c; Roth et al., 1984a). It should be noted, however, that these data may show variation depending on the source and purity of the lectins and glycoproteins and need to be estimated individually.

TABLE 1

Data for pH adjustment of colloidal gold and lectins or glycoproteins for complex formation. Modified from Roth (1983c)

	pH
Ricinus communis lectin I	8.0
Ricinus communis lectin II	8.0
Peanut lectin	6.3
Helix pomatia lectin	7.4
Soybean lectin	6.1
Lens culinaris lectin	6.9
Lotus tetragonolobus lectin	6.3
Ulex europeus lectin I	6.3
Bandeirae simplicifolia lectin	6.2
Mannan from *Candida utilus* or *Saccharomyces cerevisa*	7
Horseradish peroxidase	7.2-8.0
Ovomucoid	4.8
Fetuin	5.4
Cholera toxin	6.9
Tetanus toxin	6.9



Lectin-gold complexes are very stable and retain their specific bioactivity for long periods of time. Such preparations can be easily applied in direct, so-called one-step labeling techniques. For various reasons another type of technique has been worked out, the cytochemical affinity technique which is a two-step technique. This principle was first described by Bernhard and Avrameas (1971) and involves the use of appropriate glycoproteins in a second incubation step for interaction with already tissue bound, unlabeled lectin molecules. At present horseradish peroxidase and ovomucoid-gold complexes (Geoghegan and Ackerman, 1977) are used for interaction with Concanavalin and wheat germ agglutinin, respectively, and fetuin-gold (Roth et al., 1984a) for the Limax flavus lectin.

The application of these reagents in light microscopy is possible on routinely fixed and embedded tissues (Figs. 1 and 2). Fixation with (para-)formaldehyde, glutaraldehyde or mixtures of both gave very satisfactory results, but Bouin's or Carnoy's fluid cannot be recommended since nonspecific binding reactions have been observed (Roth, 1983a). Incubations can be performed on deparaffinized and rehydrated paraffin sections (Roth, 1983b) or frozen sections. Since the section thickness is a limiting factor for structural resolution the use of semithin (0.5-1.5 µm) sections from Epon (Fig. 3) or Lowicryl K4M embedded tissues provides superior results (Lucocq and Roth, 1984, 1985; Roth et al., 1984a). Epon removal before the cytochemical reactions is performed according to the procedure described by Maxwell (1978). Further details about the concentration of the lectin-gold or glycoprotein-gold complexes, the duration of the incubation steps and the cytochemical control reactions can be found in previous publications from our laboratory (Roth, 1983b; Roth et al., 1984a; Lucocq and Roth, 1984, 1985). The cytochemical reaction on such sections can be seen by the appearance of a pink to red coloration of the positive structures. This color is due to the reflection of transmitted visible light by colloidal gold and is not produced as the result of a revealing procedure. In addition to bright field transmitted light illumination sections can be observed by phase contrast and Nomarski differential interference contrast.

The details for the use of the reagents to detect sugar residues on thin sections from only aldehyde-fixed and subsequently low temperature Lowicryl K4M embedded tissues can be found in our earlier publications (Roth, 1982, 1983a,b; Roth et al., 1984a). Such embedding conditions have been demonstrated to preserve the structure and conformation of cellular and extracellular glycoconjugates to such an extent that their reactivity with a whole spectrum of lectins with different sugar binding specificities was retained (Roth, 1983b; Roth et al., 1984a). At the same time cellular fine structure was preserved to a high degree which allowed precise correlation between the marker colloidal gold and the labeled cellular structures (Figs. 4 and 5).

Fig. 3. Semithin section from Epon embedded rat kidney stained with Helix pomatia lectin-gold complex. All epithelia along a longitudinally sectioned cortical collecting duct are positive. Adjacent cross-sectioned proximal convoluted tubules are also intensely stained.

Fig. 4. Ultrathin section from Lowicryl K4M embedded rat kidney incubated with Helix pomatia lectin-gold complex. Part of an intercalated cell of a cortical collecting duct is shown. Gold particle label is found along the plasma membrane and over numerous cytoplasmic vesicles.

Fig. 1. Paraffin section from rat kidney labeled with Helix pomatia lectin-gold complex. Proximal and distal convoluted tubules are intensely stained.

Fig. 2. Paraffin section from rat kidney labeled with Lotus tetragonobulus lectin-gold complex. Only capillaries are stained but not the proximal and distal convoluted tubules.

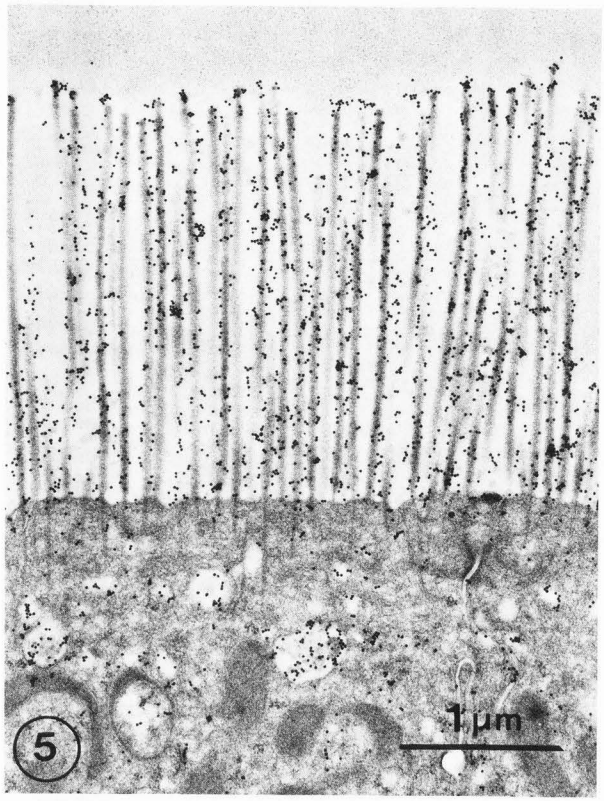
Studies on glycocalyx domain formation and cell type specific glycocalyx compositional differences

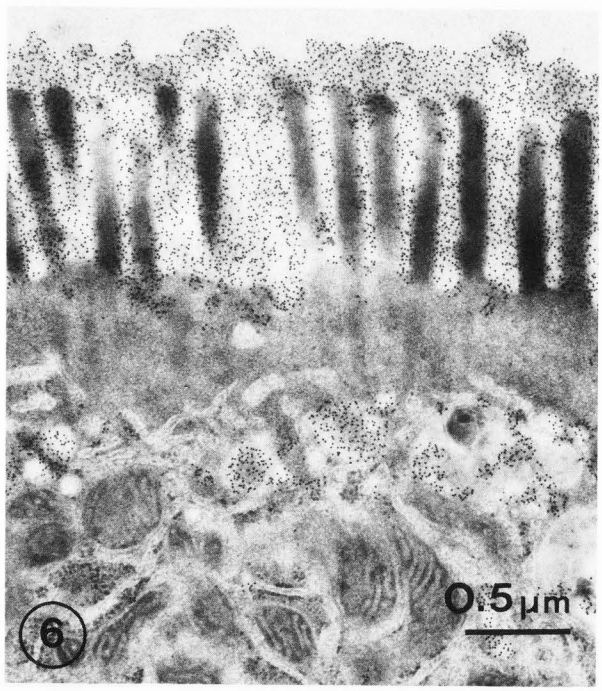
The surface of animal cells is rich in carbohydrates, and quantiative as well as qualitative differences in the nature of this surface coat, the glycocalyx, have been detected in different epithelial cell types with a variety of staining techniques (Winzler, 1970; Martinez-Palomo, 1970; Rambourg, 1971; Spicer et al., 1981). In the following results obtained with two different lectins in rat kidney will be presented and discussed. We used the Helix pomatia lectin which specifically recognizes terminal non-reducing N-acetyl-D-galactosamine residues (Hammarström and Kabat, 1969) and the Limax flavus lectin with its extraordinary specificity for N-acetyl and N-glycolylneuraminic acid (Miller et al., 1982; Ravindranath et al., 1985).

In the kidney glomerulum a highly specialized cell type, the podocyte, forms the visceral epithelium of Bowman's capsule. From their cell bodies originate long foot processes which come into contact with the glomerular basement membrane. Based on the shape of the podocytes, two different plasma membrane regions are classically distinguished: (i) the free surface which includes the cell body and the foot processes above the slit diaphragm, and (ii) the base of the foot process below the slit diaphragm and in close contact with the lamina rara externa of the basement membrane. In the intact glomerulum, binding sites for the Helix pomatia lectin-gold complex were found only over the glomerular basement membrane and here the gold particles appeared preferentially distributed over the lamina rara externa and the lamina densa with very little label appearing over the lamina rara interna. In addition to single gold particles, often groups of gold particles were seen. In many instances, the particle clusters were closely associated with the plasma membrane of the foot process base from which they extended to varying depths into the lamina rara externa and lamina densa. A quantitative evaluation of the distribution of gold particle label showed that about 93% of the total gold particle label was confined to the lamina rara externa and the lamina densa. None of the other glomerular structures including the free surface of the podocytes was labeled. In other experiments we processed isolated glomerular basement membranes and found no label with Helix pomatia lectin-gold complex. Altogether, these data in concert with published biochemical data about the carbohydrate composition of the basement membrane led us to conclude that with this lectin a component of the glycocalyx was detected rather than a basement membrane component. Therefore, it seemed that the glycocalyx of the foot process base is a highly specialized domain in that it is the only region of the podocyte surface to contain cytochemically demonstrable terminal N-acetyl-D-galactosamine residue.

The labeling pattern for sialic acid residues along the podocyte plasma membrane as detected with the Limax flavus lectin was in striking contrast to the Helix pomatia lectin labeling pattern (Charest and Roth, 1985). Upon quantification of the distribution of the gold particle label we found that the plasma membrane of the podocyte body was the most intensely labeled region (about 60%), followed by the plasma membrane of the foot processes above the slit diaphragm (about 25%), whereas the plasma membrane of the foot process base was only weakly labeled. Based on this remarkably regionalized distribution of sialic acid residues, three plasma membrane regions could be defined. Apparently, the sialic acid recognized by the lectin is mainly on the oligosaccharide chains of the major glomerular sialoglycoprotein, podocalyxin, which was described by Kerjaschki et al. (1984). It should be stressed that although we have observed striking differences in the composition of the glycocalyx in different regions of the podocyte surface we do not know at present how these specific patterns are generated nor how they are maintained and most interestingly what their functional meaning is.

Our investigations on the epithelial cells lining the lumen of the urinary tubules have also revealed the existence of glycocalyx specializations depending on the cell types considered. For example, the intercalated cells in the collecting ducts exhibited a differential binding for the Helix pomatia lectin (Brown et al., 1985). The plasma membrane of intercalated cells present in the cortex and the outer stripe of outer medulla labeled intensely with the lectin (Figs. 3 and 4), but were negative or only weakly labeled in the inner stripe and inner medulla. These results indicate that a morphological identical cell type in terms of its glycocalyx composition represents a heterogeneous population in different regions of the collecting duct. With the help of the Limax flavus lectin we could reveal the existence of another type of glycocalyx specialization along the urinary tubule (Roth and Taatjes, 1985). Sialic acid residues were detectable in the plasma membrane of the epithelia of proximal and distal tubules as well as the limbs of loop of Henlé. The thick ascending limbs of Henlé's loop, however, were for the most part free of label. Whereas in the medullary portion of the thick ascending limbs all cells were negative, in the cortical portion microvilli-bearing cells were found to be positive. As already mentioned above, at present the functional meaning of these cell type related glycocalyx specializations is unclear but it is tempting to speculate that they might be related to the specialized functions fulfilled by these different tubular regions.





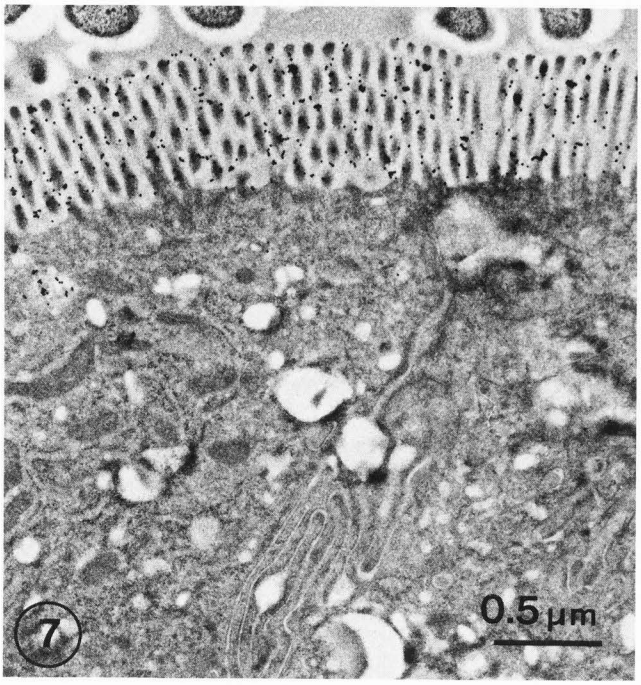


Fig. 5. Demonstration of terminal N-acetyl-D-galactosamine residues in the brush border of duodenal absorptive enterocytes with Helix pomatia lectin-gold complex. Label is also present over multivesicular bodies and cytoplasmic vesicles. Lowicryl K4M thin section.

Fig. 6. Demonstration of blood group A substance in the brush border of duodenal absorptive enterocytes with a monoclonal antibody which also stains the mucus layer adhering to the microvilli and cytoplasmic vesicular structures. Lowicryl K4M thin section.

Fig. 7. Demonstration of the monoganglioside G_{M1} in duodenal absorptive enterocytes. The gold particle label is restricted to the brush border region and absent from the baso-lateral plasma membrane domain. Lowicryl K4M thin section.

Studies on subcellular location of glycosylation steps

Glycosylation is the process by which most cellular proteins acquire covalent bound sugars (Kornfeld and Kornfeld, 1985). The sugar is transferred from a donor sugar nucleotide to the acceptor oligosaccharide by the action of specific glycosyltransferases. A few of such glycosyltransferases have been localized by immuno electron microscopy (Roth and Berger, 1982; Roth et al., 1984b, 1985; Dunphy et al., 1985). However, immunolocalization of a glycosyltransferase does not allow to make conclusions about its enzymatic activity. But the combination of glycosyltransferase immunolabeling with lectin labeling provides direct information for the site at which the glycosyltransferase exerts its in vitro function. We have studied one terminal glycosylation step, the

addition of sialic acid to N-linked glycoproteins, in intestinal goblet cells (Roth et al., 1984a, b) and in hepatocytes of intact liver (Roth et al., 1985). Sialic acid residues as detected with the Limax flavus lectin - fetuin-gold technique (Roth et al., 1984a) became first detectable in thiamine pyrophosphatase positive trans Golgi apparatus cisternae. In addition, they were also found in a complex structure which was continuous with the Golgi apparatus cisternal stack, the trans-tubular network. The trans-tubular network exhibited in addition to thiamine pyrophosphatase activity also acid phosphatase activity. In hepatocytes, the distribution of immunolabel for sialyltransferase mirrored the Golgi apparatus distribution of sialic acid residues. These findings led us to conclude that sialylation occurs in this complex Golgi apparatus structure composed of trans cisternae and trans-tubular network.

The use of lectin Concanavalin A which binds to terminal and internal mannose and glucose residues allowed us to visualize early events of N-glycosylation which are characterized by the co-translational en bloc transfer of a lipid-linked preassembled oligosaccharide, $Glc_3Man_9GlcNAc_2$, and its post-translational trimming. From biochemical work it can be anticipated that most trimming which results in removal of the glucose residues and some of the mannose residues takes place in the endoplasmic reticulum. Indeed, labeling with Concanavalin A was almost restricted to the smooth and rough endoplasmic reticulum in hepatocytes and intestinal absorptive cells and sparse label was present over cis elements of the Golgi apparatus but absent from medial and trans elements (Roth, 1983a, b).

The subcellular localization of terminal N-acetyl-D-galactosamine residues with the Helix pomatia lectin-gold complex has provided some more information about the sites of synthesis of O-glycoproteins in intestinal goblet cells (Roth, 1984). The assembly of the oligosaccharide chains of O-linked glycoproteins is initiated by the transfer of N-acetyl-D-galactosamine to a serine or threonine residue which is followed by the addition of other sugars. The synthesis of such oligosaccharide chains is often terminated by the addition of N-acetyl-D-galactosamine thereby yielding blood group A activity. Terminal N-acetyl-D-galactosamine residues were not detectable in the endoplasmic reticulum of the intestinal goblet cells but were found in the Golgi apparatus, mucin droplets and the plasma membrane. Over the Golgi apparatus, labeling was observed over cis and trans cisternae but not over medial cisternae. The labeling over cis Golgi apparatus cisternae was speculated to indicate the subcellular site of onset of O-glycosylation. On the other hand, the labeling over trans cisternae was interpreted to represent the terminal step in the synthesis of blood group A active oligosaccharides. The latter assumption was substantiated by the immunolocalization of the blood group A transferase (Roth et al., 1984b).

Studies with monoclonal anti-carbohydrate antibodies

The production of monoclonal antibodies against well defined carbohydrate epitopes has been repeatedly reported in the literature (Kannagi et al., 1983; Hakomori et al., 1983; Nilsson et al., 1985), but with the exception of a few light microscopical studies they have not yet found application in electron microscopic studies. In Fig. 6 the results obtained with a monoclonal antibody against blood group A substance are shown. Thin sections from low temperature Lowicryl K4M embedded duodenum of a blood group A subject were incubated with this monoclonal antibody. After a further incubation step with affinity-purified rabbit anti-mouse IgG, protein A-gold was applied. The intermediate antibody incubation step was necessary because of the weak reactivity of the mouse monoclonal antibody with protein A. These incubation steps resulted in an intense labeling of the brush border of absorptive enterocytes and the adhering mucus layer. Furthermore, the labeling of cytoplasmic vesicles in the apical region of this cell can be seen in the photograph. Although we have tested so far only one carbohydrate specific monoclonal antibody, it can be anticipated that at least some of them could become useful probes for detection of carbohydrates on Lowicryl K4M sections by electron microscopy.

Studies with toxins

Bacterial toxins such as cholera and tetanus toxin bound to particles of colloidal gold have been used to investigate retrograde neuronal transport (Schwab and Thoenen, 1978) and receptor-mediated endocytosis (Ackerman et al., 1980; Montesano et al., 1982). Cholera and tetanus toxin bind specifically to the oligosaccharide portion of gangliosides. A cholera toxin-gold complex was applied to study the sites of its binding on human intestinal eptithelial cells (Roth, 1985). At the same time this procedure should provide information about the distribution of the ganglioside G_{M1}, which is the receptor for this toxin. In the plasma membrane of enterocytes, label for cholera toxin receptor was found in the brush border region but was absent in the basolateral plasma membrane (Fig. 7). This result provides another example of glycocalyx specialization which is related to cell polarity.

Studies using glycosyltransferases

Glycosyltransferases are a group of enzymes catalyzing the synthesis of specific

oligosaccharides by transfer of a monosaccharide from a sugar nucleotide donor substrate to an acceptor substrate (Beyer et al., 1981). Because of their strict acceptor specificities glycosyltransferases have been shown to be valuable reagents in the *in vitro* elucidation of oligosaccharide structures. Previous electron microscopical studies with various lectins (Roth, 1983b, d; Roth et al., 1983) and neuraminidase (Roth et al., 1984a) have shown that oligosaccharide structures are exposed on ultrathin sections of Lowicryl K4M embedded tissues. It appeared likely, therefore, that glycosyltransferases could also be used to detect sugar moieties of oligosaccharides in ultrathin tissue sections. Based on the pioneering work of Hill and coworkers (Sadler et al., 1979), Lucocq et al. (1987) have recently applied galactosyltransferase to detect N-acetyl-D-glucosamine residues in thin sections from rat and pig liver. Purified milk galactosyltransferase and donor substrate UDP-galactose were used to galactosylate acceptor substrates in thin section. The visualization of the endogenous and tissue bound galactosyltransferase reaction product was achieved with *Ricinus communis* lectin I-gold complex. Without previous galactosyltransferase treatment the label with *Ricinus communis* lectin I-gold complex was found only in *trans* cisternae of the Golgi apparatus. After exposure to galactosyltransferase in the presence of donor substrate additional labeling was found in *medial* and *cis* cisternae. This result shows that under suitable conditions oligosaccharides residing in *medial* and *cis* Golgi apparatus cisternae and terminating in N-acetyl-D-glucosamine, can be converted to galactosylated structures to which *Ricinus communis* lectin I-gold complex binds. Already the acceptor substrate specificity of galactosyltransferase (Beyer et al., 1981; van den Eijnden et al., 1983) suggested that galactosylation should have appeared on N-linked oligosaccharides. To further substantiate this notion, thin sections after being exposed to galactosyltransferase were treated with endo-F/PNGase-F before the incubation with *Ricinus communis* lectin I-gold complex. This resulted in virtually no labeling over the Golgi apparatus. Endo-F/PNGase-F is a glycosidase which cleaves core chitobiosyl and β-aspartylglycosyl linkages of both high mannose and complex type N-linked oligosaccharides (Elder and Alexander, 1982; Plummer et al., 1984). This type of enzyme treatment together with further controls proved that galactosylation had occurred on N-linked oligosaccharides. In conclusion, it is possible to probe for and localize terminal N-acetyl-D-glucosamine containing oligosaccharides present in Lowicryl K4M tissue sections using a glycosyltransferase, namely galactosyltransferase. Most important is the observation that various endoglycosidases also can act on Lowicryl K4M thin sections providing a very useful means for further characterization of the type of galactosylated oligosaccharide. Therefore, the high specificity of glycosyltransferases can be combined with electron microscopy for high resolution *in situ* studies on ultrathin tissue sections.

Conclusions

From the data presented in this paper the following conclusions can be drawn. The embedding of aldehyde-fixed tissues with the low temperature resin Lowicryl K4M preserves the structure and conformation of various carbohydrates in the different cellular compartments so that they can be detected on thin sections by electron microscopy. For detection of cellular glycoconjugates by light and electron microscopy, ligands such as lectins, glycoproteins, monoclonal anti-carbohydrate antibodies, toxins, and glycosyltransferase can be used in conjunction with the colloidal marker system. These tools allow the visualization of glycocalyx variability in intact tissues and permit the detection of sites of intracellular glycosylation steps.

Acknowledgments

The author's work reviewed in this paper was supported by the Swiss National Science Foundation. I thank Ursula Schaub, Erica Oesch, and Daniel Wey for technical assistance.

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Editor's Note: All of the reviewers' concerns were appropriately addressed by text changes, hence there is no Discussion with Reviewers.